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# Induction of Callus from Mistletoe and Interaction with its Host Cells

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## ヤドリギ・カルスの誘導と 宿主細胞との相互作用

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### Résumé

A callus was induced from mistletoe (*Viscum album* L. var *lutescens* M.), and established on a modified Murashige-Skoog (MS) medium having a half of the ionic strength of the original MS medium and supplemented with 1.0 ppm of kinetin and 10.0 ppm of 1-naphthaleneacetic acid at 28°C under 12-hr day-length at about 2,500 lux. The callus was creamish pale pink and mainly spherical having average diameter of 7.5  $\mu$ m. The growth of the callus reached a maximum at 3 weeks incubation in PLANTEX to give about thrice amount of dry weight. The callus contained two galactose-binding lectins having molecular weights of 77,000 and 19,400 which were originally observed in the leaves of mistletoe. When the callus of mistletoe was in contact with its host beech callus, both calluses were found to grow without contact inhibition.

### 要 旨

ヤドリギ (*Viscum album* L. var *lutescens* M.) から、カイネチン1.0ppm, ナフタレン酢酸10.0ppmを含む、イオン強度を半分にしたMurashige-Skoog (MS) 改変培地を用い、28°C、12時間の明暗周期、光度約2,500 luxの条件下で培養し、カルスを誘導した。カルスは淡いピンク色で、その細胞の平均直径は約7.5  $\mu$ mであった。また、プランテックスを用いて、3週間培養したとき、その成長量が最大に達し、絶乾重量が約3倍になった。カルス細胞には、分子量77,000、19,400のガラクトース特異性レクチンが見出されたが、これらは、ヤドリギの葉にも同様に見出された。一方、ヤドリギのカルスをブナのカルスに接触させたところ、両カルスは接触阻害を起さずに生長し続けることがわかった。

### 1. INTRODUCTION

Plant cells are known to recognize each other by mutual contact and protect themselves against external intruders through the contact and following conveyance of information. A key to clarify this recognition system is expected to be obtained from

characterization of the plant host-parasite interactions. In the field of woody plant, mistletoe and its host seem to be an attractive starting materials for this line of investigation. Although the physiologically active constituents of mistletoe, its nutrient relations toward host and anatomy of mistletoe and host were reported<sup>1-12)</sup>, little is known about cell-cell interaction between mistletoe and its host. Therefore, in this study we intend to investigate the interaction between mistletoe (*Viscum album* L.) and beech (*Fagus crenata* Bl.) *in vitro*. First, we tried to induce a callus from mistletoe and confirmed the presense of the same kind of galactose-binding proteins in both leaves and callus cells of mistletoe by polyacrylamide gel electrophoresis. Then the parasite cells were contacted with host cells by placing the callus of mistletoe on that of beech as grown in the relative state and analyzed whether or not their contact inhibition occurs.

## 2. Experimental

### 2.1. Plants

Mistletoe (*Viscum album* L. var *lutescens* M.) growing on a beech at the University Forest in Ashiu, Miyama-cho, Kyoto, was harvested in May, 1988. Shoots of beech (*Fagus crenata* Bl.) growing at the Experimental Nursery, Sakyo-ku, Kyoto, was harvested in June, 1988.

### 2.2. Cell cultures

Small sections of mistletoe were excised aseptically from an apical meristem and two latest leaves and sterilized stepwisely for 30 sec with 70% ethanol, 2.5 min with 0.2% benzalkonium chloride, 5-20 min with 2% sodium hypochlorite solution and rinsed several times with sterile distilled water. Subsequently, they were transferred to 50 ml glass tube containing 25 ml of a modified solid MS (Murashige and Skoog)<sup>13)</sup> medium. It contained a half of the ionic strength of mineral salts of the original MS medium, 100.0 mg/l *myo*-inositol, 0.5 mg/l nicotinic acid and 0.5mg/l pyridoxine·HCl, 0.1 mg/l thiamine·HCl, 3% sucrose and 1% agar as listed in Table 1. Auxin and cytokinin

Table 1. Composition of the modified Murashige-Skoog medium

Compound	Final conc. (mg/l)	Compound	Final conc. (mg/l)
CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.0125	KNO <sub>3</sub>	950.0
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.1250	NH <sub>4</sub> NO <sub>3</sub>	825.0
KI	0.4150	KH <sub>2</sub> PO <sub>4</sub>	85.0
CoCl <sub>2</sub> · 6H <sub>2</sub> O	0.0125	CaCl <sub>2</sub> · 2H <sub>2</sub> O	220.0
<i>myo</i> -Inositol	100.0	MgSO <sub>4</sub> · 7H <sub>2</sub> O	185.0
Nicotinic acid	0.5	FeSO <sub>4</sub> · 7H <sub>2</sub> O	13.90
Pyridoxine · HCl	0.5	Na <sub>2</sub> EDTA	18.65
Thiamine · HCl	0.1	MnSO <sub>4</sub> · 4H <sub>2</sub> O	11.15
Sucrose	30.0	ZnSO <sub>4</sub> · 7H <sub>2</sub> O	4.30
		H <sub>3</sub> BO <sub>4</sub>	3.10

were added in various combinations. The pH of the medium was adjusted to 5.7 and the medium was then autoclaved at 120 °C for 20 min. The cultures were kept at 28 °C under 12-hr day-length at about 2,500 lux. Green and white calluses of beech were induced and subcultured in the same modified MS medium supplemented with 1.0 ppm of 1-naphthaleneacetic acid (NAA) (green callus) and 0.5 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) (white callus) together with 0.5 ppm of 6-benzylaminopurine (BA).

### 2.3. Determination of growth rate of callus

One piece of callus, average weight of 2 mg in a wet state, was planted on a fiber mat in a PLANTEX CCP-102 (TOYOBO Co., Ltd.), containing 15 ml of the medium described above supplemented with 1.0 ppm of kinetin (KIN) as cytokinin and 10.0 ppm of NAA as auxin. The PLANTEXes were sealed with air-permeable polyvinylchloride-tapes in order to prevent the microorganism contamination and loss of moisture and incubated as described above.

At weekly intervals during 4-week incubation period, calluses were blotted with a filter paper (ADVANTEC Toyo No.2) to remove moisture attached on the surface of callus, and their fresh weights were determined. The calluses were then dried until to reach constant weight in an oven at 60 °C, and the average dry weight was calculated for three samples. The growth rate was estimated as increase of dry weight of callus cells relative to the original dry weight.

### 2.4. Extraction of mistletoe proteins

The fresh leaves of mistletoe were cut into small pieces with a pair of scissors after washing with tap water followed by distilled water. Forty grams of leaves were suspended in 100 ml of 0.25mM Tris-glycine buffer (pH 8.3), and homogenized for 20 min at 5 °C. The deep-greenish suspension was centrifuged for 15 min at 8,000 × g at 5 °C to remove insoluble materials. The extracted solution was poured into 15 times volume of cold acetone. The precipitated materials were recovered by centrifugation as described above, and washed with acetone to give acetone-powder. The acetone-powder was solubilized in 0.05M Tris-HCl buffer (pH 8.7) of which NaCl concentration was 0.05M.

### 2.5. Affinity chromatography

Acid-treated Sepharose 4B was prepared by washing the gel with cold 0.2N HCl followed by shaking the suspension of the acid-washed gel (gel:0.2N HCl=1:2, v/v) at 50 °C for 3 hr<sup>(4)</sup>. The gel was washed and equilibrated with 0.05M Tris-HCl buffer (pH 8.7). Affinity chromatography was carried out by using the column (1.7 × 18 cm) of the gel at 25 °C. After application of the solubilized acetone-powder from leaves, the column was washed with the equilibration buffer until free from protein and the lectin was eluted out with the same buffer containing 0.1M lactose. The UV-positive fractions were collected, dialyzed against distilled water and lyophilized.

### 2.6 Polyacrylamide gel electrophoresis

Electrophoresis was performed in 10% (w/v) polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate (SDS) by the method of Laemmli<sup>(5)</sup>. The acetone-powder was dissolved in 0.01 M Tris-HCl buffer (pH 6.8) containing 1% SDS, 0.029 % EDTA and 5% (v/v) 2-mercaptoethanol. After the sample solutions were heated for 5 min at

100 °C, 100  $\mu$ l of each sample was applied to the gels using Malachite Green as a tracking dye. Electrophoresis was performed at room temperature initially at 1 mA/gel, then at 2mA/gel after the bands were moved into the separation gel. The calibration kit (Pharmacia) was used as the marker proteins. After electrophoresis, the gel was stained for proteins with 0.25% Coomassie Brilliant Blue R-250 and destained with 10% acetic acid.

### 3. Results and Discussion

#### 3.1 Induction and growth of callus from mistletoe

When induction of callus from mistletoe was tried using the MS medium containing various concentrations and types of plant hormones, all green-colored transplanted mistletoe turned into brown within one day. Since this seemed to be caused by the high ionic strength in the original MS-medium, as frequently observed in the cases of woody plants<sup>16)</sup>, next we tried to induce callus in the modified MS medium having a half of ionic strength of the original one. Preliminary findings indicate that addition of 1.0 ppm of BA kept the transplanted mistletoe green for three months without growth and induction of callus. On the other hand addition of 1.0 ppm KIN was found to be favorable for the growth of mistletoe callus. Based on these results, the induction of callus from mistletoe was carried out under various combinations of hormones. After 4-week incubation at 28 °C, only one callus could be induced from the leaf under a combination of 1.0 ppm of KIN and 10.0 ppm of NAA as shown in Fig. 1.

The residual tissues either became dead or were contaminated by microorganisms during the culture. The callus has been easily subcultured in the same medium for more than 2 years at interval of about 3 weeks. The calluses are creamish pale pink in color and mainly spherical in shape having average diameter of 7.5  $\mu$ m as shown in Figs. 2a and 3.

When effects of 2,4-D were analyzed on the growth of the mistletoe callus cells, the callus was found to grow at the concentration below 10.0 ppm in the presence of 1.0 ppm of KIN. However, the color of the callus at 0.05 ppm and 0.1 ppm of 2,4-D became darker on their surface, and changed into reddish pink at higher than 0.5 ppm. Similarly, the mistletoe callus could grow on the same modified MS media supplemented with 0.5 ppm of BA and 0.5 ppm of 2,4-D or 1.0 ppm of NAA which were used for beech callus, although the growth rate is not so fast as that given under the combination of KIN and NAA. The mistletoe callus grown on the medium containing 0.5 ppm of BA and 0.5 ppm of 2,4-D is shown in Fig. 2b. The adaptation of mistletoe callus to the growth condition of beech callus will be described later. In conclusion, the callus grew most at 10.0 ppm of NAA and 1.0 ppm of KIN supplemented in the half of ionic strength of MS medium. The change in relative growth rate of the callus after subculture on this hormonal condition is shown in Fig. 4, which indicates that the growth reached maximum after 3 weeks to give about thrice amount of dry weight. In Fig. 4 the growth curves of green and white beech calluses were included for comparison. The growth rate of the mistletoe callus cells was in the intermediate between these beech calluses.

The present results indicate that PLANTEX has two advantages to measure the growth rate of callus cells: one does not have to use agar system, and it is very easy

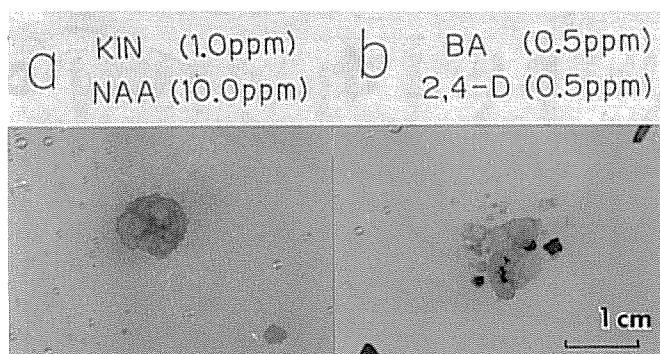


Fig. 2 Callus growth of mistletoe.  
Callus in 2 weeks after subculture in PLANTEXes under the hormonal conditions described in the figure.

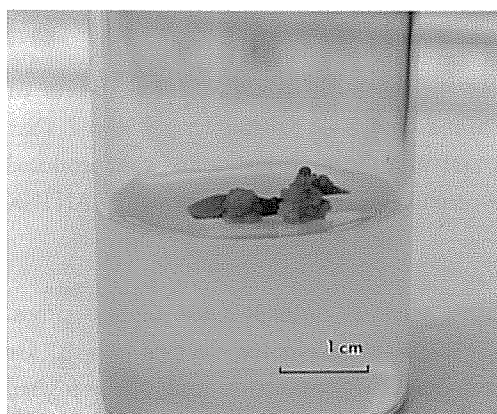


Fig. 1 Induction of callus of mistletoe.  
Induced callus in 3 weeks after transplantation of a small portion of mistletoe with an apical meristem and two leaves on the modified MS medium supplemented with 1.0 ppm of KIN and 10.0 ppm of NAA. Two calluses are induced from the leaves.

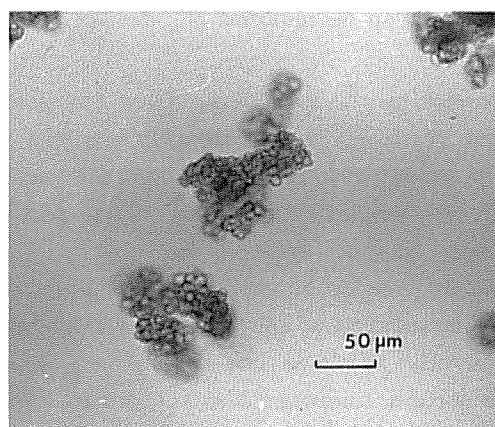


Fig. 3 Microphotograph of callus cells of mistletoe.

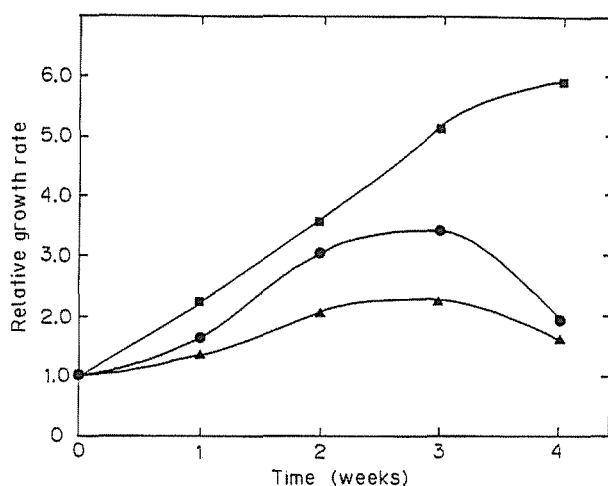


Fig. 4 Growth curves of callus cells of mistletoe and beech in PLANTEX: mistletoe callus (●) grown on the modified MS medium supplemented with 1.0 ppm of KIN and 10.0 ppm of NAA, green beech callus (▲) on the modified MS medium supplemented with 0.5 ppm of BA and 1.0 ppm of NAA, white beech callus (■) on the modified MS medium supplemented with 0.5 ppm of 2,4-D.

to pick up the callus cells from the fiber mats inside the PLANTEX. The merits of the PLANTEX was thoroughly reported by Yajima and Taki<sup>17)</sup>, and they showed that the growth rate of some callus cells was enhanced and more anthocyanin was produced from a beefsteak plant callus. The present study provided us the new usage of PLANTEX in the field of tissue culture.

### 3.2 Detection of lectins in the mistletoe callus

The extracts of mistletoe have been used against various diseases since ancient times. Recently mistletoe has been shown to contain D-galactose-binding lectins which are important components in the extract, and their chemical properties and physiological activities were investigated<sup>9-11)</sup>. Based on these results the presense of the same lectins in the mistletoe callus cells is expected to be used as a useful index for identification of the cells. In order to ascertain this, we analyzed the soluble proteins of both mistletoe and its callus. At first, the mistletoe lectin was isolated by an affinity chromatography on acid-treated Sepharose 4B gels. A typical result is shown in Fig. 5. The subunit structure of the lectins recovered from the fractions eluted with 0.1 M lactose was analyzed by sodium dodecyl sulfate gel electrophoresis (in the presence of 2-mercaptoethanol) and the results are shown in Fig. 6. Three minor and three major proteins designated as a to c and d to f respectively could be detected in the fraction bound to the acid-treated Sepharose 4B gels. The callus cells were found to contain two proteins corresponding to a and f components, whereas two major galactose binding proteins, d and e, were not detected in the callus cells. This difference may be due to the fact that the pathway of the protein synthesis is changed after induction of the callus.

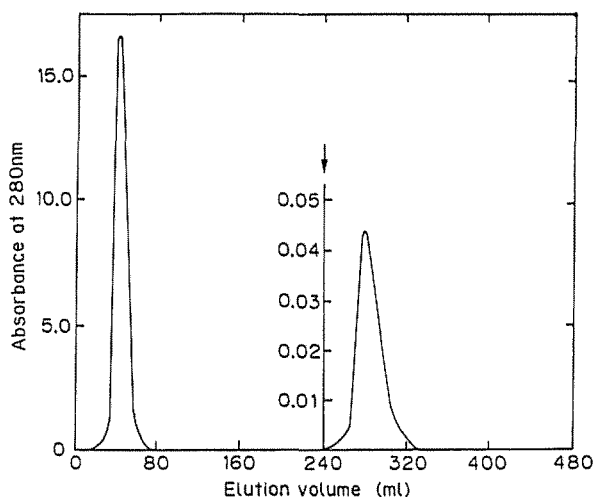


Fig. 5 Affinity chromatography of mistletoe galactose binding lectins on acid-treated Sepharose 4B.

Acetone powder from 40 g of mistletoe leaves was applied on a column ( $1.7 \times 18$  cm). The lectins were eluted with 0.05 M lactose in 0.05 M Tris-HCl buffer (pH 8.7). The arrow indicates the change of the elution conditions.

### 3.3 Interaction between both calluses of mistletoe and beech

Regarding the recognition mechanisms between plant cells, it seems to be quite interesting to investigate what happens when host and parasite cells come in contact with each other. In order to carry out this experiment *in vitro*, it should be necessary to culture the host and parasite callus in the same medium. The green and white beech calluses were subcultured in the same modified MS medium that was used for mistletoe callus, but unfortunately they were grown on the different hormonal conditions. Since transplantation of the beech callus into medium of the mistletoe was found to be difficult and the mistletoe callus had wide adaptability for different hormonal conditions, the mistletoe calluses were in turn adapted for the conditions of the beech callus cells. The results clearly indicate that the mistletoe calluses could actually grow on the same media

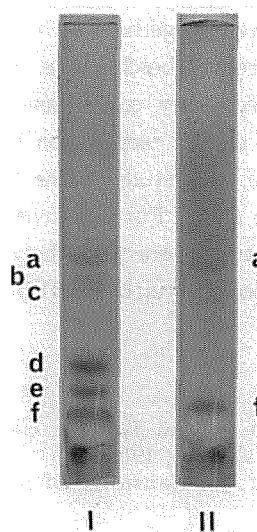


Fig. 6 SDS-Polyacrylamide gel electrophoresis of galactose-binding lectins from mistletoe (I) and callus cells (II).

Electrophoresis was performed in 10% polyacrylamide gels and gels were stained with Coomassie Brilliant Blue R-250 as described in the text.



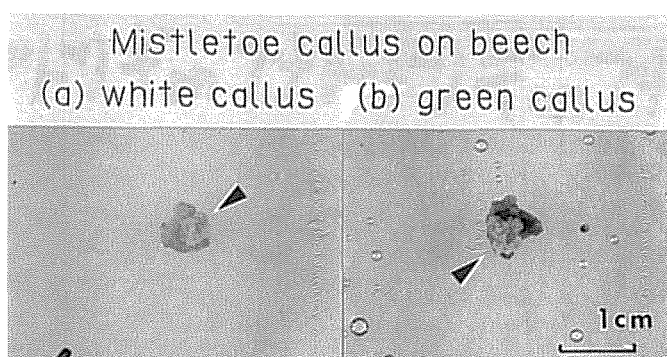


Fig. 7 Growth of mistletoe callus cells on green (a) and white (b) beech callus. After adaptation of the growth conditions, the mistletoe calluses were put on the callus cells of beech and cultured for 2 weeks as described in the text. Mistletoe calluses are indicated by the arrows.

in which the beech calluses grew in the PLANTEX. Figure 2b shows the adaptation of mistletoe to the condition of the white beech callus. Therefore, after subcultured for 3 weeks, a small piece of the mistletoe callus was transplanted on top of the beech callus, and incubated at 25°C.

As shown in Fig. 7, no contact inhibition occurred in both cases, and the mistletoe calluses grew together with the beech ones. However, the mistletoe callus did not intrude into the beech callus, and the contact between two calluses was not so strong as we expected. They could easily be separated by using tweezers. Although a detailed discussion on the recognition between the host and the parasite callus is difficult to investigate, this study is the first attempt to contact the host and parasite mistletoe calluses *in vitro*. Further investigations for functions of galactose-binding lectins in mistletoe and interactions between the mistletoe and beech cells are now in progress by using suspension-cultured calluses.

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